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Nuclear Shuttling in Sporadic Breast Cancer as a Therapeutic
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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Agents targeting DNA double strand break repair (DSBR) deficiency, such as PARP1 inhibitors, are highly selective in killing BRCA1-mutated breast tumors, and the toxicity is minimal in mouse models. However, more than 90% of breast cancers are sporadic, which carry wild-type (wt) BRCA1 and are proficient in DSBR. BRCA1 is a nuclear shuttling protein, which regulates homologues recombination (HR)-mediated DSBR when nuclear and enhances apoptosis when cytoplasmic.
BARD1 retains BRCA1 in the nucleus, whereas ionizing radiation (IR) induces p53-dependent export of BRCA1 to the cytosol. This research project is to test the hypothesis: whether targeting BRCA1 from the nucleus to the cytoplasm will render sporadic breast cancer cells become defective in DSBR and enhance apoptosis. Furthermore, if the combination of induced repair deficiency and augmented apoptosis will render sporadic breast cancers become highly susceptible to selective killing by PARP1 inhibitors.
We found that (1) both IR treatment and transient expression of small peptide tr-BRCA1 induce cytoplasmic translocation of BRCA1 protein and subsequent transient inhibition of HR-mediated repair of DSBs in human breast cancer cells in vivo and in mouse tumor model; (2) importantly, we demonstrated that this transient tBRCA1 cytosolic translocation and suppression of repair of DSB correlated with the significantly increased cytotoxic response of breast cancer cells to PARP1 inhibition *in vitro*. We are still in the processes of verify this important findings in vivo tumor models; (3) finally, p53 status, which controls BRCA1 translocation in response to IR also plays important role in IR-induced sensitization of breast cancer cells to PARP1 inhibition.

15. SUBJECT TERMS
BRCA1 DSBR PARP1 Breast Cancer

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Final Progress report

Rendering DNA Repair Defective by Targeting Wild-type BRCAI Nuclear Shuttling

Introduction:

BRCA1 is a nuclear shuttling protein, which regulates homologues recombination (HR)-mediated DSBR when nuclear and enhances p53-independent apoptosis when cytoplasmic. BARD1 retains BRCA1 in the nucleus, whereas ionizing radiation (IR) induces cell cycle-independent export of BRCA1 to the cytosol. Both BARD1 and IR induce BRCA1 shuttling via the CRM1/exportin pathway. It is hypothesized that targeting BRCA1 from the nucleus to the cytoplasm will render cells defective in DSBR and enhance apoptosis. The combination of induced repair deficiency and augmented apoptosis will render sporadic breast cancers highly susceptible to selective killing by agents targeting DNA DSB lesions. The purpose and scope of this research project is: to determine if targeting BRCA1 from the nucleus to the cytosol will compromise DSBR and result in a pro-apoptotic environment which renders tumor cells susceptible to PARP1 inhibitor-induced cytotoxicity using both BRCA1-proficient human breast cancer cell lines and mouse breast tumor models.
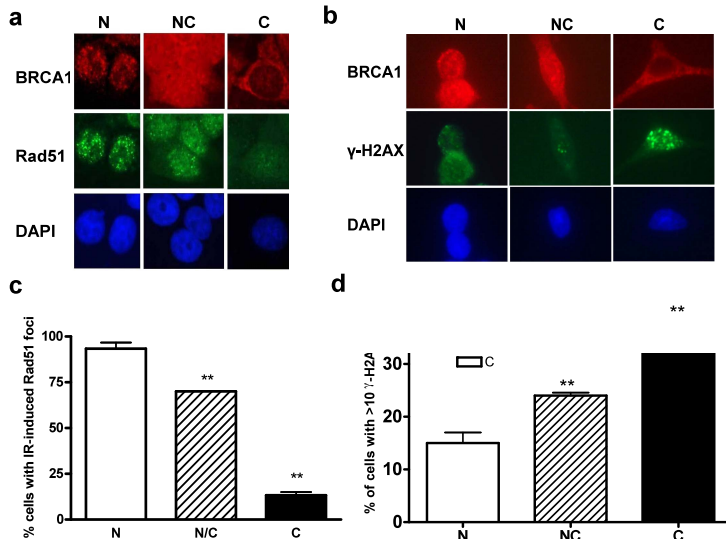

Fig. 1. Homologous recombination (HR)-mediated repair of DSB requires nuclear BRCA1. Rad51 foci, an *in vivo* functional marker of HR activity, and γ -H2AX foci, an *in situ* marker of DSBs, are shown in relation to BRCA1 subcellular localization in MCF7 cells following 3Gy radiation. BRCA1 localization is categorized as nuclear only (N), cytosolic only (C), or nuclear/cytosolic (N/C) (**a**) Representative immunocytochemistry staining of BRCA1 (red), Rad51 foci (green), and nucleus (blue). (**b**) Same staining as in (a), except with γ -H2AX foci (green). (**c**) Percentage of cells expressing Rad51 foci as a function of BRCA1 localization. IR-induced Rad51 foci are found predominantly in cells with BRCA1 present in the nucleus (76%), suggesting that HR is strongly associated with nuclear BRCA1. (**d**) Percentage of cells expressing γ-H2AX foci as a function of BRCA1 localization. Radiation-induced γ -H2AX foci are found predominantly in cells with BRCA1 present in the cytosol (38%), suggesting that the defect of DSB repair is strongly associated with cytosolic BRCA1. **p<0.001

Body:

Aim 1: To determine if IR-induced BRCA1 cytosolic accumulation will inhibit DSBR, promote apoptosis, and sensitize breast cancer cell to PARP1 inhibitor.

Result from Aim 1:

1. HR-mediated DNA DSB repair requires BRCA1 nuclear localization: We and others have demonstrated that BRCA1 is a nuclear shuttling protein[10, 11] whose function is regulated by its subcellular localization. We found that DNA-damage-induced Rad51 nuclear foci, an *in vivo* marker of HR activity, is seen predominantly in cells with nuclear BRCA1, but rarely in cells with cytosolic BRCA1 (**Fig. 1a and 1b).** This suggests that HR activity is suppressed when BRCA1 is relocated in the cytosol. Consistent with this, γ -H2AX foci, which are an *in situ* marker of DSBs, are seen predominantly in cells with cytosolic BRCA1 but rarely in cells with nuclear BRCA1 (**Fig. 1c and 1d**). These data suggest that the function of BRCA1 in the repair of DSBs is dependent on its

nuclear localization. Nuclear export of BRCA1 protein is sufficient to inhibit its function and induce HR-mediated DSB-repair deficiency, which results in an accumulation of DSBs.

2. BRCA1 nuclear export (NE): BRCA1 protein is exported from the nucleus to the cytosol through the CRM1/exportin pathway[11, 12]. The nuclear export of BRCA1 is regulated by its association with other proteins. Consistent with previous reports by Scully and Livingston[13], we found that ionizing radiation (IR) induces BRCA1 nuclear foci formation at DNA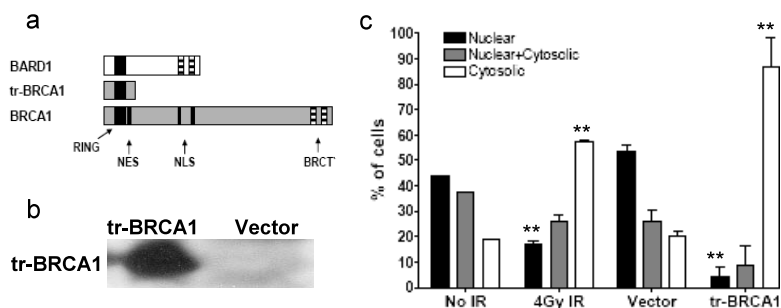 damage sites within 1 hour. Interestingly, we found that IR also induces cell-cycle–independent BRCA1 nuclear export at later time points. This IR-induced BRCA1 NE is a transient and reversible process that peaks at 24–48 hours[10] and returns to basal level after 60–72 hours (data not shown). We have demonstrated that the tumor suppressor p53, which interacts with BRCA1[14], promotes IR-induced BRCA1 nuclear export[10]. For cells deficient in p53, BRCA1 accumulates in the nucleus and is resistant to IR-induced NE[10]. This intriguing observation implies that BRCA1 may function differently in early- versus late-phase response to DNA damage. It is plausible that nuclear export of BRCA1 may be a mechanism

Fig. 2. (**a**) Schematic of BRCA1, BARD1, and tr-BRCA1. (**b**) expression of tr-BRCA1 in MCF7 cells. **(c)** Cells were treated with 3Gy radiation or transfected with tr-BRCA1 or vector control. Four hours after radiation or 24 hrs after transfection, cells were subjected to immunofluorescence staining for BRCA1 location. **p<0.001

employed to regulate both DNA repair in the nucleus and cell death in the cytosol following DNA damage. In contrast to the role of p53, the BRCA1-associated ring domain protein (BARD1) prevents BRCA1 nuclear export and retains BRCA1 in the nucleus[12]. BARD1 inhibits BRCA1 nuclear export through its binding to the N-terminal ring domain region of BRCA1, thereby masking the BRCA1 nuclear export signal (NES) and blocking BRCA1 from interacting with CRM1/exportin[12, 15, 16]. Transient expression of the small peptide tr-BRCA1, a truncated form (1–301aa) of BRCA1 that contains the NES and BARD1 binding activity, can release wt-BRCA1 from BARD1 and effectively shift BRCA1 to the cytosol in both p53-proficient[17] (see **Fig. 2**) and p53-deficient cells (data not shown).

3. Depletion of nuclear BRCA1 impairs HR-mediated DSB repair: To confirm that HR-mediated DSB repair function is dependent on nuclear BRCA1 and to determine if depletion of nuclear BRCA1 would compromise BRCA1-dependent and HR-mediated repair of DSBs, we assessed the effect of transient expression of tr-BRCA1 on the HR-mediated and BRCA1-dependent DSB repair capacity using MCF7 cells carrying a chromosomally integrated HR reporter substrate DR-GFP[17]. A site-specific DNA DSB can be induced by the transient expression of the I-*Sce*I endonuclease in MCF7/DRGFP cells[17]. The sequence of the HR substrate is designed such that only HR-mediated repair of the I-SceI-induced DSB can restore GFP function. Expression of GFP in cells can then be quantified using flow cytometry. As shown in **Fig. 3**, transient expression of tr-BRCA1 significantly decreases the percentage of GFP-positive MCF7 cells compared to control MCF7
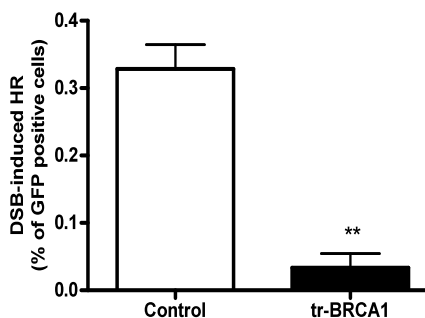

Fig. 3. Decreased HR capacity of MCF7 cells transiently expressing tr-BRCA1. MCF7 cells, which express the DRGFP HR repair substrate, were co-transfected with tr-BRCA1 or vector control and I-SceI-expression plasmid or empty vector. 48 hours later, cells were subjected to flow cytometric analysis for GFP expression, indicative of HR activity. **p<0.001

cells (0.33% versus 0.03%, p<0.001), resulting in a 10- fold decrease in HR efficiency[17]. Thus, these data suggest a dependence of HR on nuclear BRCA1. Furthermore, they show that transient expression of tr-BRCA1 is a valid method to manipulate BRCA1 function in breast cancer cells.

4. Correlation of IR-induced BRCA1 NE with cell susceptibility to PARP1 inhibition (PARPi): To determine the effect of IR-induced "BRCA1ness" on cell susceptibility to PARPi, the cytotoxic response to PARPi was measured in LNCAP prostate cancer cells and MCF7 breast cancer cells. Both cell lines are p53-proficient and respond to IR-induced BRCA1 NE. On the other hand, p53-deficient PC3 prostate cancer cells fail to respond to IR-induced BRCA1 NE. IR-induced BRCA1 NE was assessed by the reduction in the proportion of cells with nuclear BRCA1 after IR, which were: 62% to 20% in LNCAP, 42% to 22% in MCF7, but only 47% to 43% in PC3).
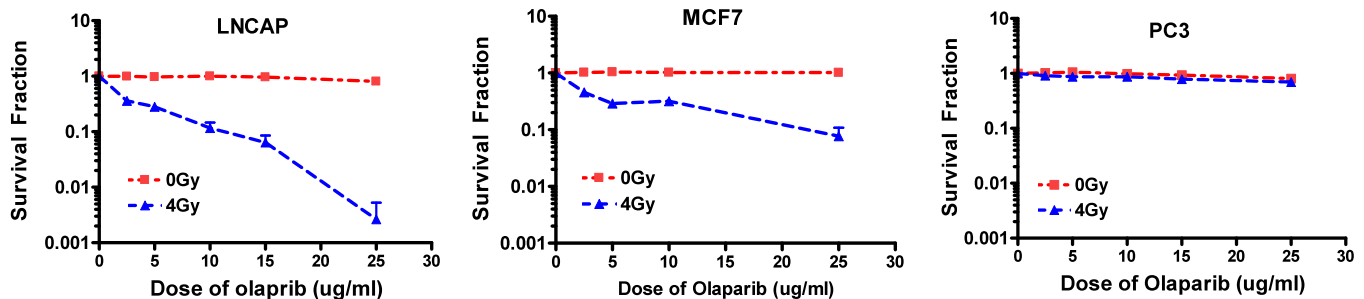
Fig. 4. IR-induced BRCA1 nuclear export is associated with IR-induced sensitization of sporadic cancer cells to PARPi. Cells were exposed to Olaparib alone, or 4Gy of IR 24 hours prior to treatment with Olaparib. Cytotoxicity was measured by colony formation assay. Cytotoxicity from IR + Olaparib was normalized by the survival fraction from 4Gy IR alone.

The cytotoxicity from Olaparib alone, IR alone, or IR + Olaparib was assessed using a colony formation assay. As shown in **Fig. 4,** all three cell lines are resistant to Olaparib alone. Interestingly, IR pretreatment resulted in a dramatic increase in cell killing (10-100 folds) in LNCAP cells and MCF7 cells (both of which are p53-functional and efficient in IR-induced BRCA1 NE (**Fig. 4**)), but had no effect in PC3 cells (which are p53-nonfunctional and resistant to IR-induced BRCA1 NE). The data suggest that IR-induced and p53-dependent export of BRCA1 from the nucleus to the cytosol may be a key determinant IR-induced sensitization of wt BRCA1 cancer cells to PARPi.

Aim 2: To determine if targeting BRCA1 from the nucleus to the cytosol by disrupting BARD1 function by expression of the small peptide tr-BRCA1, a truncated form (1-301aa) of BRCA1 that contains the NES and BARD1 binding activity, will result in sensitization of breast cancer cell to PARP1 inhibitor.

Results from Aim 2:

Transient expression of the small peptide tr-BRCA1, a truncated form (1-301aa) of BRCA1 that contains the NES and BARD1 binding activity, can release wt-BRCA1 from BARD1 and effectively shift BRCA1 to the cytosol[14] (see **Fig. 3 and oral presentation in appended oral presentation**).

Key Research accomplishments:

Oral presentation at American Society of Therapeutic Radiation Oncology (ASTRO) annual meeting 2010

Oral presentation at Radiation Research Society annual meeting 2010

Reportable outcome:
Oral presentation at American Society of Therapeutic Radiation Oncology (ASTRO) annual meeting 2010
Oral presentation at Radiation Research Society annual meeting 2010

Conclusion: This research aims to develop novel therapeutic strategies to make sporadic breast cancer cells that are proficient in BRCA1-dependent and homologous recombination (HR)-mediated DSB repair susceptible to PARPi therapy by transiently making them phenotypically BRCA1 deficient by relocating BRCA1 from the nucleus to the cytoplasm using either IR or by expressing a small peptide tr-BRCA1. If this proves to be successful, the use of PARP1 inhibitors could be expanded not only to the majority of breast cancer patients, but also to any solid tumor. Furthermore, the knowledge gained from this investigation will be a platform for discovery and development of small-molecule–based drugs that can be used as systemic treatment to create synthetic lethality to PARPi via the transient induction of BRCA1 NE and DSB-repair deficiency in many solid tumors. Successful implementation of such a strategy would lead to a paradigm shift in the development of therapeutic strategies in cancer treatment.

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Targeting BRCA1 localization to convert tumor cell susceptibility to poly (ADP-ribose) polymerase-1 (PARP1) inhibition
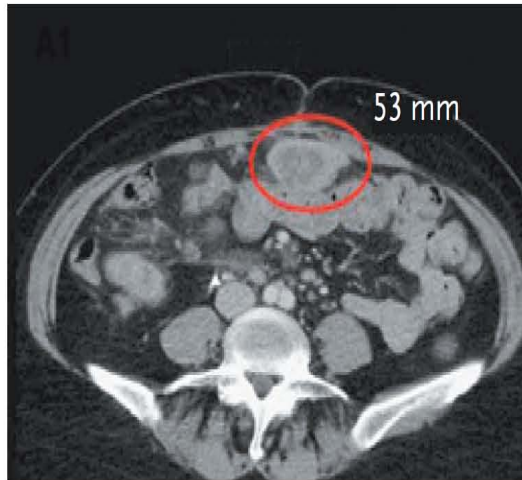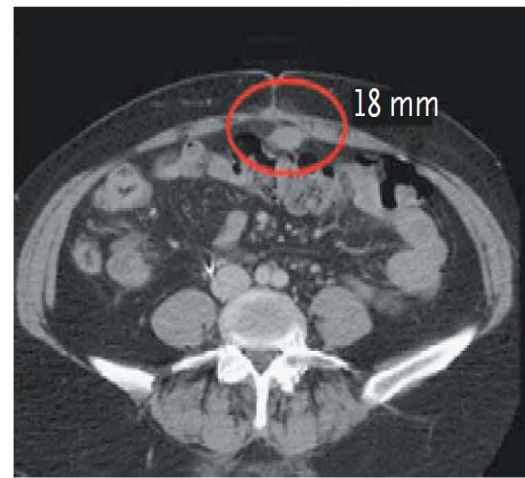
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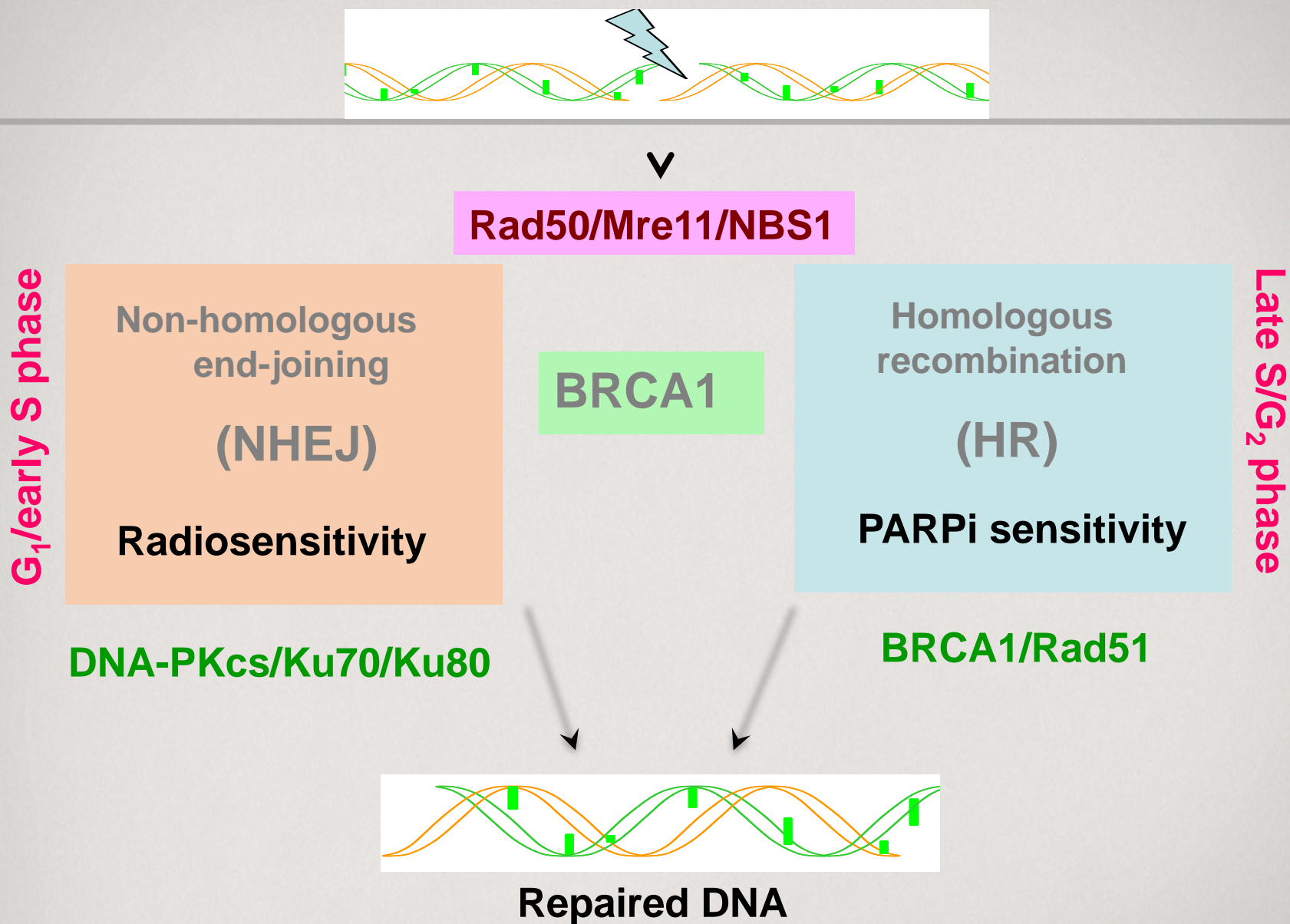
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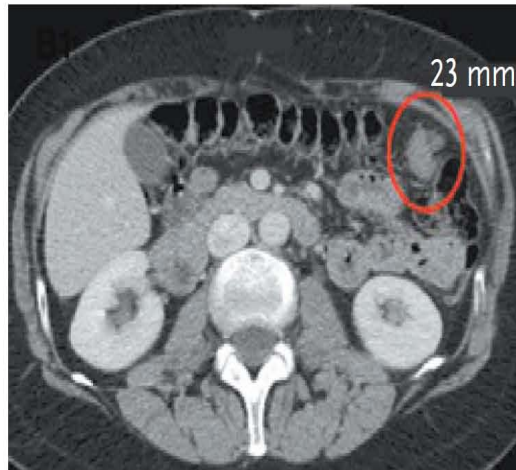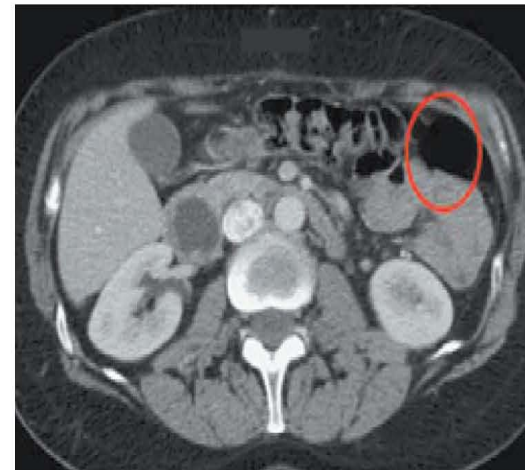

Fong et al. NEJM 361:2009

PARP inhibition and tumor-selective synthetic lethality

γH2AX

SSB

PARP1 inhibitor

DSBs from collapsed DNA replication forks

sporadic cancer
Normal BRCA1/2
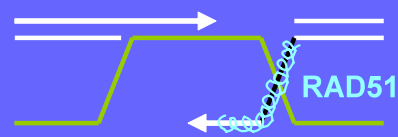
Familial cancer
mutated BRCA1/2

RAD51

HR-based repair

Impaired HR repair

Cell Survival

Cell Death

Rendering DNA repair defective by targeting BRCA1 nuclear export

Nuclear/cytoplasmic shuttling of BRCA1 controls HR-mediated DSB repair
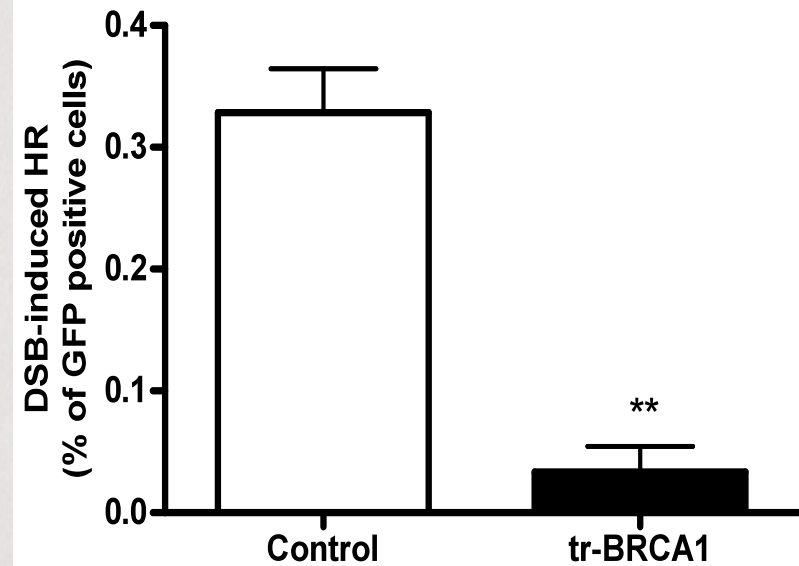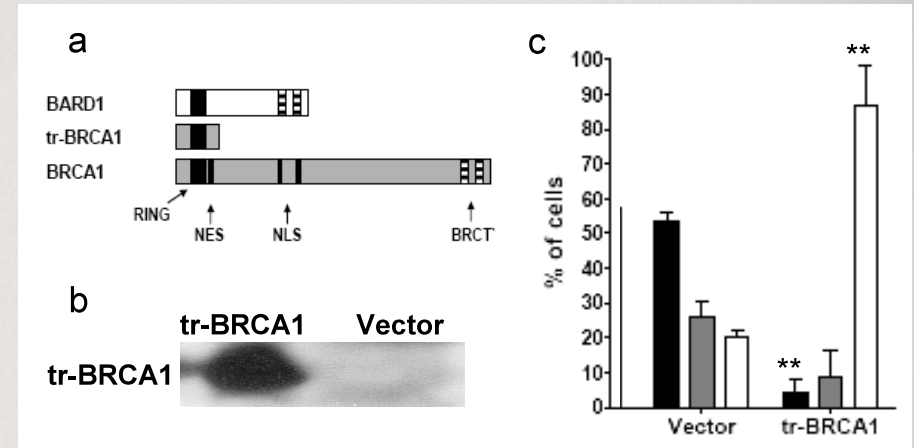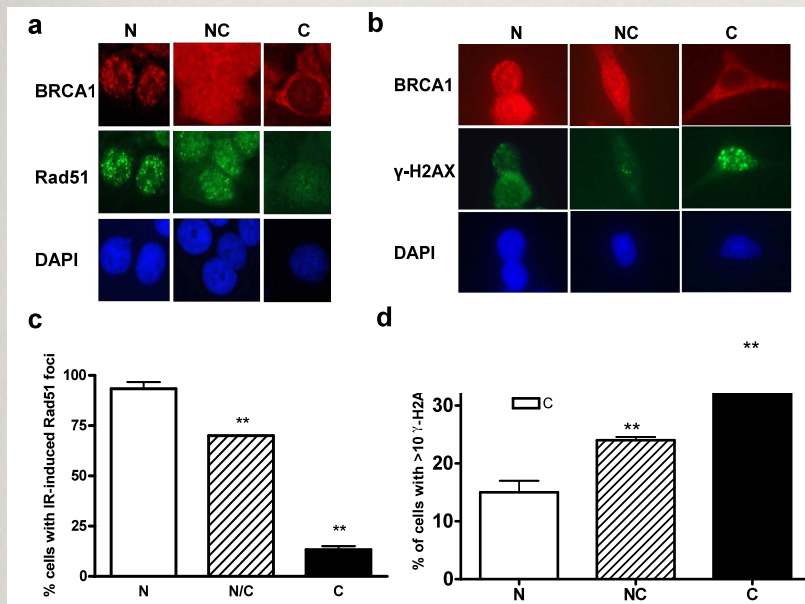
Radiation-induced BRCA1 cytoplasmic translocation is dependent on p53
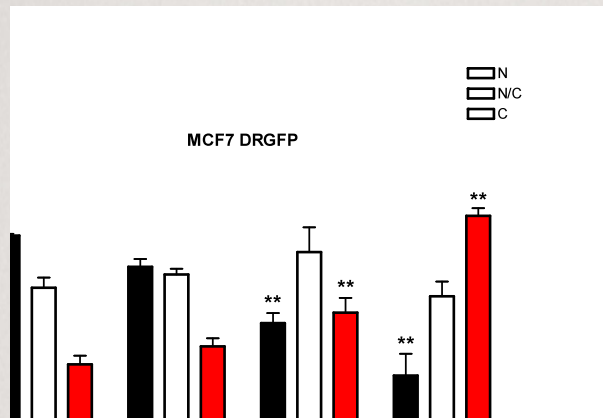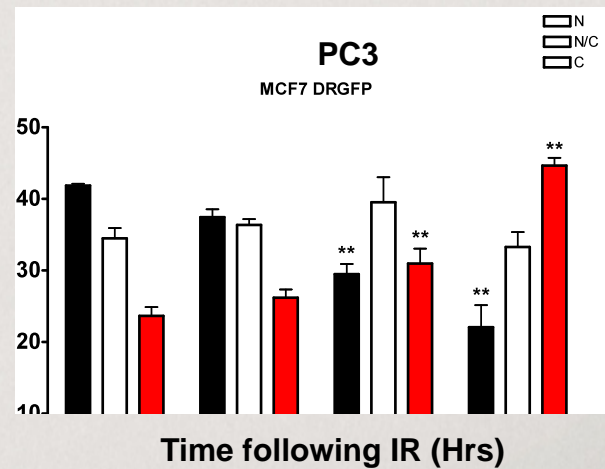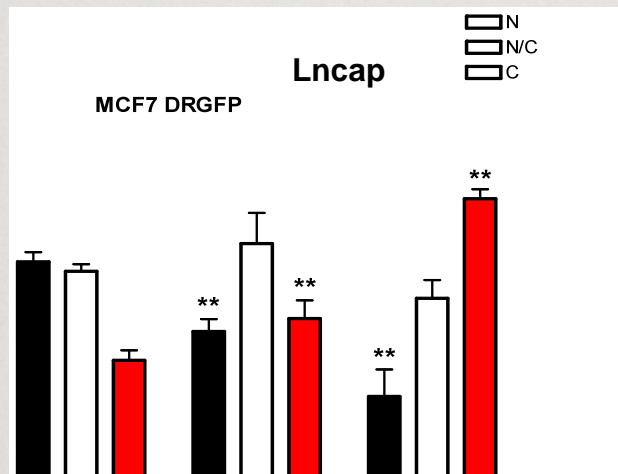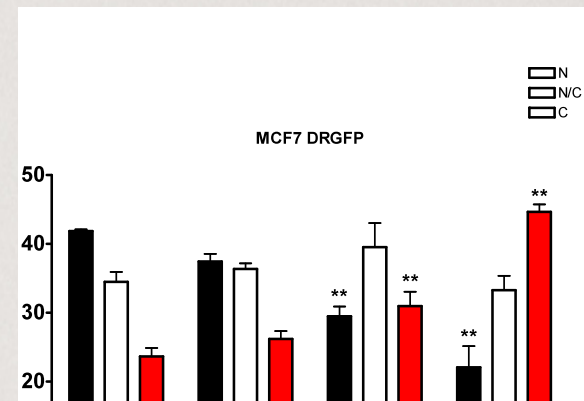
tr-BRCA1 induces BRCA1 export from the nucleus to the cytosol regardless p53 status
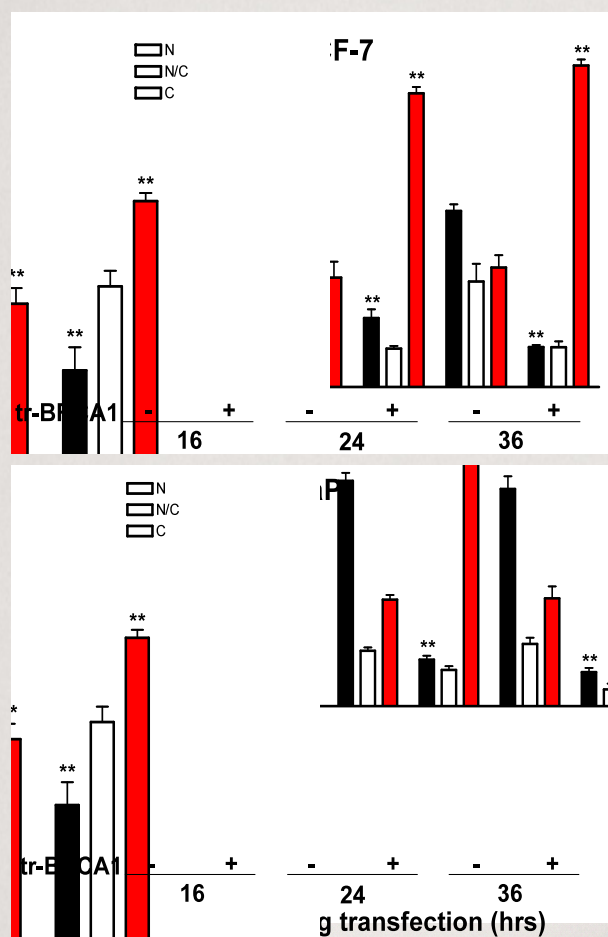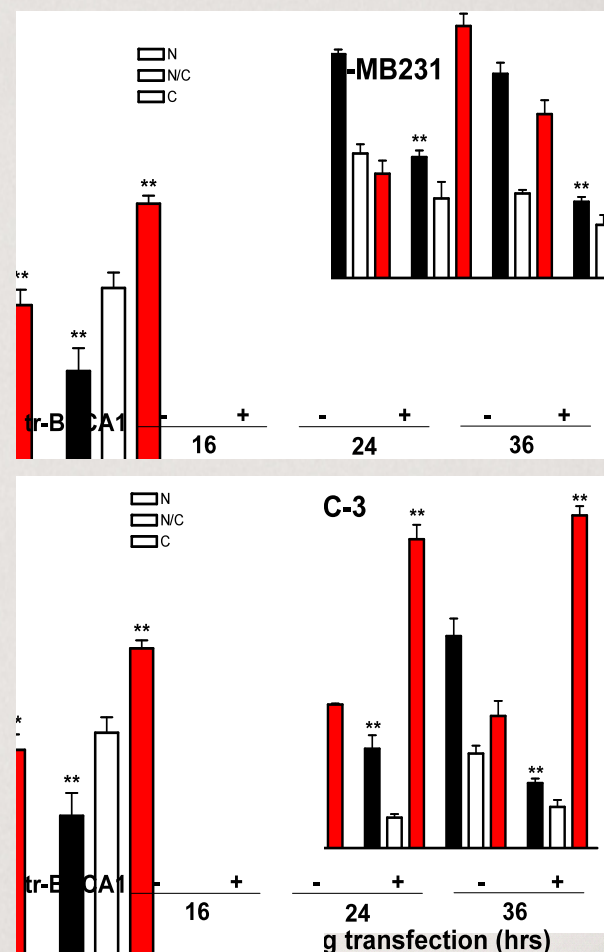
Targeting BRCA1 to the cytosol with tr-BRCA1 inhibits HR-mediated repair of DSBs
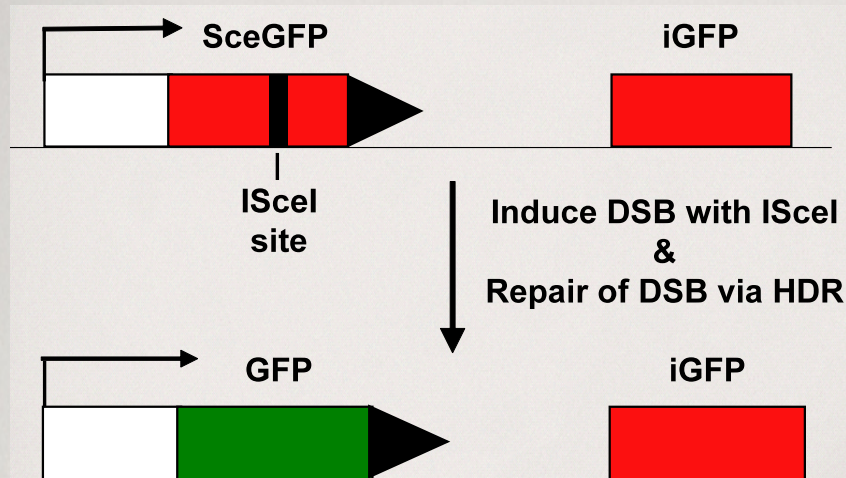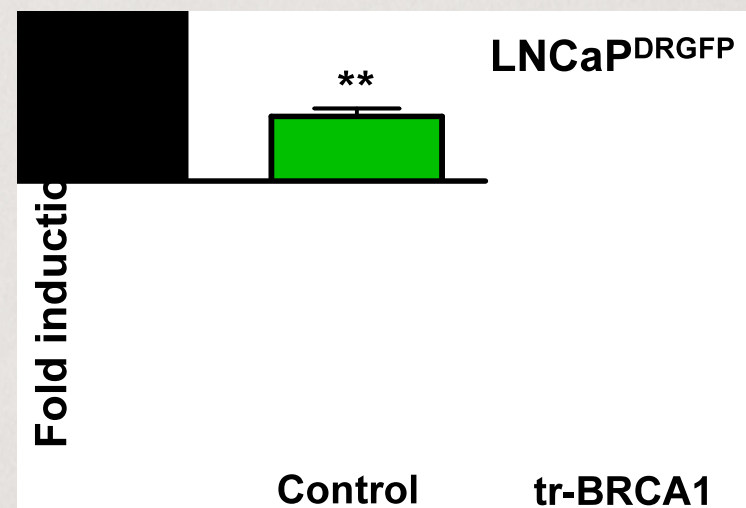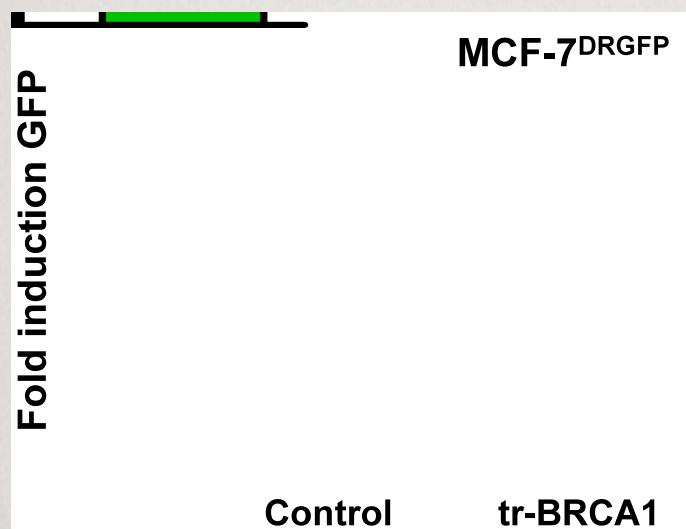
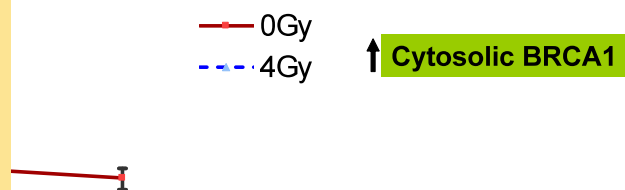# To Convert tumor cell to be susceptible to Olaparib by targeting BRCA1 to cytosol
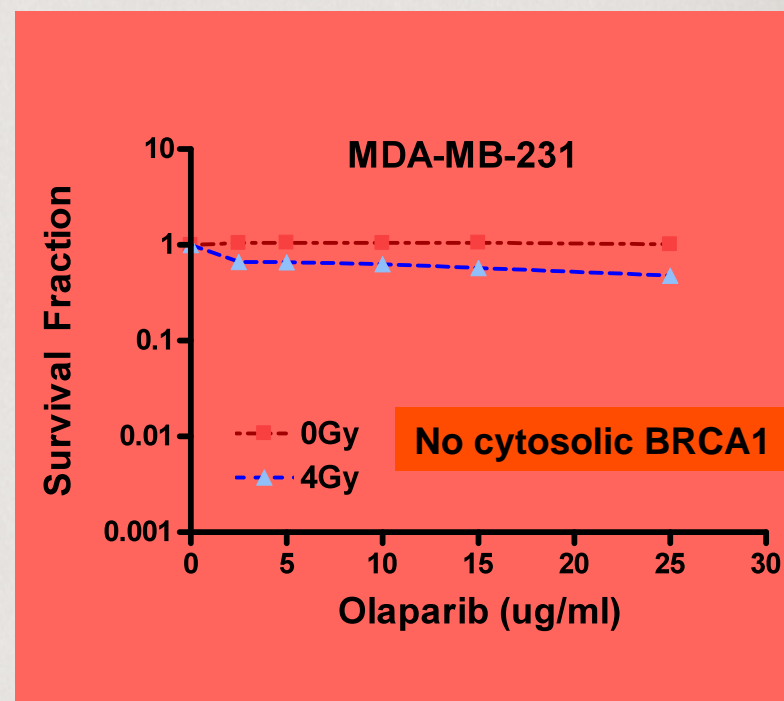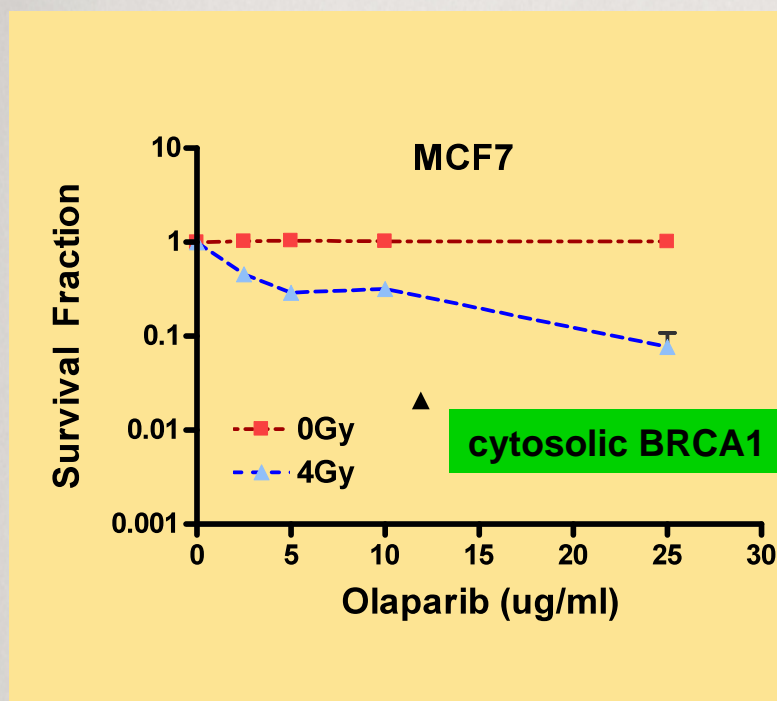
Summary

- **Targeting BRCA1 localization away from its nuclear repair substrate with radiation or tr-BRCA1 can transiently induce HR repair defect, which subsequently convert sporadic tumor cells to become susceptible to PARPi**

- **This strategy may also be feasible for other types of solid tumors, including Lung, brain, and pancreases cancers**

Vanderbilt-Ingram Cancer Center